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TREATMENT WITH AN ASCOCHLORIN DERIVATIVE, AS-6 INCREASES $^{45}Ca^{2+}$ BINDING ON THE PLASMA MEMBRANE OF ADIPOCYTES IN db/db MICE

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Summary: Genetically obese diabetic mice (db/db) have greatly diminished $^{45}Ca^{2+}$ binding on the plasma membranes of the adipocytes (45 - 55%) compared with their lean littermates. Treatment for 1 week with a diet admixture of AS-6 (0.1% in the diet) significantly restored the binding to a level comparable to the lean littermates. The addition of AS-6 in vitro had no effect on the binding, which eliminates the possibility that AS-6 is a Ca^{2+} ionophore. The results suggest that AS-6 treatment enhances the Ca^{2+} binding by causing structural alteration(s) in the membranes.

Calcium ion (Ca) mediates many types of coupling between extracellular stimuli and subsequent cellular responses in mammalian cells (1). However, there is no direct evidence that Ca acts as a second messenger in insulin action (2). But lack of such evidence does not rule out the possibility that Ca is involved in insulin action (3). It is increasingly evident that Ca mediates some cellular events following insulin binding.

The genetically obese diabetic mouse (db/db) is an excellent model for human adult onset diabetes with insulin resistance (4). We have previously shown that oral treatment with an ascochlorin derivative AS-6 ameliorates the diabetic syndrome in db/db mice by reducing partially the insulin-resistance in the whole animal, and at the tissue and cellular levels (5-7). Our subsequent studies indicate that the plasma membranes of db/db adipocytes are both structurally and functionally abnormal compared with their lean littermates (8-10). Particularly, phosphorylation of the membrane proteins at 55K and 57K was less sensitive to calcium in the db/db mice than in the lean

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mice. In the present study we evaluated the difference in Ca binding on the membranes in db/db and their lean littermates, and showed how AS-6 treatment affects the binding in the db/db mice.

MATERIALS and METHODS

Animals: Male db/db mice and their lean littermates 12 weeks old were supplied by the Chugai Pharmaceutical CO., Tokyo and male ddY mice 8 weeks old by the Shizuoka Experimaental Animal Cooporation. The db/db mice were randomly allocated to 2 groups (n = 6) soon after arrival, and AS-6 treatment was initiated immediately. For 1 week one of the db/db group together with the lean littermates (n = 6) were fed a control pellet diet (CE-2, Nihon CLEA, Tokyo). The other group fed an AS-6 admixture (0.1% in CE-2) of diet pellets. All mice were allowed free access to diet and water throughout the study. Under this condition, the treatment significantly ameliorates the diabetic syndrome of db/db mice, decreasing usually the serum glucose by 25-50%, the triglyceride by 45-60% and the immunoreactive insulin by 20-40%. The diet intake is not affected by the treatment, resulting in slightly more weight gain than the untreated controls.

Preparation of partially purified plasma membranes: All of the procedures were the same as reported previously (9). Briefly, the mice were killed after being treated for 1 week and the epididymal adipose tissues were removed. The adipose tissues of each group were combined, cut into small pieces and digested with collagenase by the method of Rodbell (11). Partially purified plasma membranes were prepared from the epididymal adipocytes according to the method of Jarett (12). Protein was determined by the method of Lowry et al. (13).

$^{45}Ca^{2+}$ binding (14): The plasma membranes (50-65 µg as protein) of adipocytes from AS-6 treated and untreated db/db, and the lean mice were added at 37°C with 50 mM Tris-HCl buffer (pH 7.4) and 0.2 µci $^{45}Ca^{2+}$ (Amersham International, England) with or without insulin or AS-6. The total volume of the reaction mixture was 1 ml. The mixture was incubated at 37°C for 2-20 min, then the reaction was terminated by the addition of ice cold 10 mM EDTA. The mixture was centrifuged at 20000 g for 20 min, the pellets were twice washed with 10 mM EDTA, and the radioactivity of the pellets was determined.

RESULTS

Fig 1 demonstrates the time-dependence of $^{45}Ca^{2+}$ binding on the membranes. In each group the binding was increased 2.2- to 2.3-fold in 18 min of incubation compared with that for 2 min. When the binding was expressed as the radioactivity bound per mg of protein in the membranes (the upper Figure), the binding was slightly greater in the db/db controls (16-23%) than in the lean littermates, but the increase was nonsignificant. AS-6 treatment significantly enhanced $^{45}Ca^{2+}$ binding over both the db/db controls (24-28%) and the lean mice (48-52%). When the binding was expressed as the radioactivity bound per g of adipose tissue, however, the db/db controls bound only 44-53% as much $^{45}Ca^{2+}$ as the lean mice did. This decrease was clearly due to the enlargement

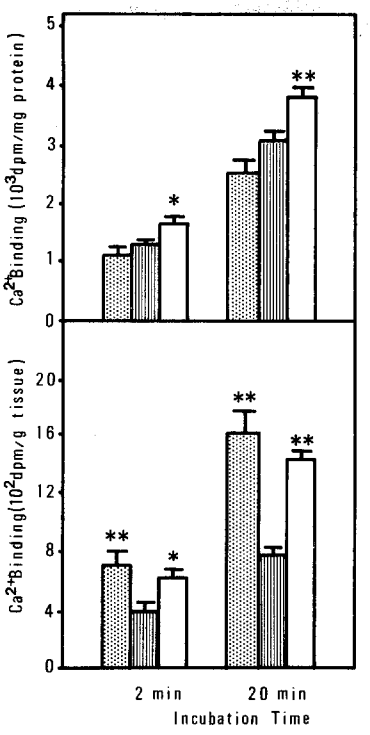

Fig 1. $^{45}Ca^{2+}$binding to the plasma membranes of adipocytes from AS-6 treated and untreated db/db mice and their lean littermates. The bars represent the mean + SE (n = 4). *P < 0.05 and **P < 0.01, statistical significance from the untreated db/db controls in the unpaired t-test. The group was consisted of each 6 mice. The lean littermate group: The db/db controls: The AS-6 treated db/db group: The plasma membranes (50-65 µg as protein) were incubated at 37°C with 50 mM Tris-HCl (pH 7.4) and 0.2 µci $^{45}Ca^{2+}$in a total volume of 1 ml (14). Quadruplicate tubes were used for the assay. After incubation the reaction was terminated by the addition of ice cold 10 mM EDTA, and centrifuged at 20,000 g for 20 min. The pellets were twice washed with the Tris buffer. The radioactivity of pellets was determined by a liquid scintilation counter.

of adipocytes which resulted from increased fat storage (15). On the g tissue weight basis, AS-6 treatment recovered $^{45}Ca^{2+}$binding to the levels comparable to that of the lean littermate level.

Fig 2 shows the effect of insulin on $^{45}Ca^{2+}$binding in vitro. Again, the difference in the binding was apparent between the AS-6 treated and untreated db/db groups; the treatment increased the basal binding by 33% ($P < 0.05$) and the binding in the presence of insulin by 42% ($P < 0.05$). But insulin had no effect on the binding either with or without AS-6 treatment.

Partially purified plasma membranes were prepared from the adipocytes of non-diabetic ddY mice and the effect of insulin and AS-6 on $^{45}Ca^{2+}$binding was

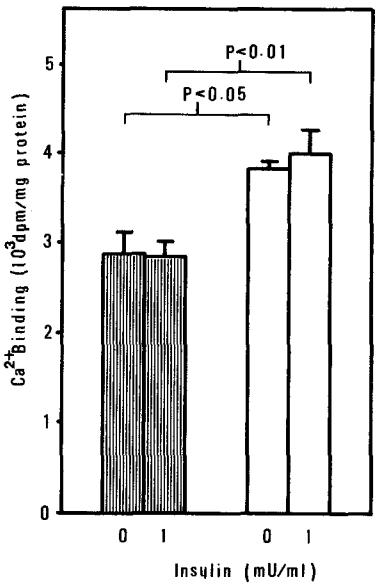

Fig 2. Effect of insulin on the binding of $^{45}Ca^{2+}$ to the adipocyte plasma membranes
Only db/db mice were used in this experiment. The symbols are the same as in Fig 1. One group (n = 6) was fed the control diet pellets, CE-2, and the other group (n = 6) fed a diet admixture of AS-6 (0.1% in the CE-2). On day 7 all of the mice were killed by ether anesthesia and the plasma membranes were prepared as described in the text. The plasma membranes (50-65 µg as protein) were incubated at 37°C for 20 min in the mixture of Tris-HCl buffer (50 mM, pH 7.4) and 0.2 µci $^{45}Ca^{2+}$ with or without insulin (1 mU/ml, bovine crystalline insulin, Sigma). After 20 min the reaction was terminated by the addition of ice cold EDTA (10 mM) and the mixture was centrifuged at 20,000 g for 20 min. Then, the pellets were twice washed with the Tris buffer. The radioactivity of pellets was determined by a liquid scintilation counter.

determined in vitro. Like the membranes from the diabetic mice, insulin at 1 mU/ml had no effect on the binding on the nondiabetic membranes. The binding fluctuated upon the addition of AS-6 (1-5 µg/ml) but the changes were not statistically significant from the control levels (Table 1).

DISCUSSION

The role of calcium in insulin action is controversial. The hypothesis that insulin triggers the release of Ca into cytosol from the cell organella is ruled out, since the binding of insulin to its receptors does not elevate cytosolic Ca in the various cell types. However, it is clear that insulin changes Ca fluxes (3). Recently, a new hypothesis has been proposed by Eckel and Reinauer (16) as well as Williams and Turtle (17) that insulin displaces

Table 1. The effects of insulin and AS-6 on $^{45}Ca^{2+}$ binding to the adipocyte plasma membranes in vitro (Mean ± SE)

Addition	Concentration	Ca^{2+} binding
	mU/ml	dpm/mg protein
Insulin	0	1225 ± 171
	1	1269 ± 160
	μg/ml	
	0	821 ± 71
AS-6	1	774 ± 43
	5	901 ± 100

The figures represent the mean ± SE (n = 4). The addition of insulin and AS-6 produced no significant changes in $^{45}Ca^{2+}$ binding capacity of the membranes.
Epididymal adipose tissues were removed from male non-diabetic ddY mice 8 weeks old. The adipose tissues were cut into small pieces and digested with collagenase by the method of Rodbell (11). The adipocytes were twice washed with the homogenization buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.25 M sucrose) and homogenized in 3.5 volumes of the buffer with a teflon homogenizer. The plasma membranes were prepared by differential centrifugation as described by Jarett (12). All assays were done quadruplicate.

Ca bound to plasma membranes thus altering affinity of the receptors. This hypothesis is supported by three lines of evidence: (i) when myocytes from adult rats are exposed to EDTA, the insulin binding and 3-O-methylglucose transport are greatly decreased below the controls, and this decrease is partially restored by the addition of divalent cations calcium and magnesium (16); (ii) the divalent cations as well as sodium increase insulin binding to the solubilized receptors from rat adipocytes (18); and (iii) the plasma membranes from insulin-treated rat adipocytes bind significantly more insulin than the controls, whereas the addition of insulin to the membranes has no effect on the binding (19). According to the hypothesis the more the exchangable Ca that binds to the membranes, the more responsive to insulin the cells become. In support of this hypothesis the following evidence has been presented; (a) streptozotocin-induced diabetic (stz-diabetic) rats become deficient in Ca due to poor absorption from the duodenum (20), (b) the cardiac sarcolemmal membranes from stz-diabetic rats greatly decrease Ca binding compared with nondiabetic controls (14), (c) conversely, calmodulin was

elevated in various tissues from diabetic mice (21,22). It is important that most of these abnormalities are restored to normal by treating the animals with insulin.

As reported previously, the adipocytes from db/db mice decrease insulin binding to 1/4-1/10 as much as that of the lean littermates and glucose transport becomes virtually insensitive to insulin action (9). Oral treatment with AS-6 partially restores the insulin binding. Our subsequent studies on the plasma membranes prepared from the adipocytes reveal that the membranes are apparently abnormal compared with the lean. Functionally, insulin reduces phosphate incorporation into the db/db membranes below the basal level but in the lean this does not occur (8). Ca greatly stimulated phosphorylation of the membrane proteins at 55K and 57K in the lean, but those of db/db are relatively insensitive to Ca. In addition, the db/db membranes incorporate less arachidonic acid into phosphatidylinositol than the lean, and AS-6 treatment trippled the incoporation. These abnormal functions suggest that the membranes are defective in the processes in which Ca participates.

The Ca binding per g of adipose tissue was significantly less in the db/db mice than in the lean littermates, as shown in this study. Oral treatment with AS-6 restored the binding to levels comparable to the binding in lean littermates, even when it was expressed on the basis of g of adipose tissue. The results are similar to that of McDonald et al.(19), who demonstrated that the plasma membranes of insulin-treated rat adipocytes bound significantly more Ca than the controls. However, it remains to be evaluated why the treatment enhances Ca binding to the membranes. It is likely that an increase in Ca binding produced by AS-6 links to an increase in insulin binding and a subsequent enhancement of glucose metabolism in the adipocytes of db/db mice.

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